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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF)
)
Hans Deckmyn and Nancy Cauwenberghs)
)
SERIAL NO: 10/049,868)
)
FILED: February 11, 2002)
)
FOR: Cell Lines, Ligands and Antibody Fragments)
For Use In Pharmaceutical Compositions for)
Preventing and Treating Haemostasis)
Disorders)

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Dear Sir:

Under the International Convention, for the purposes of priority, applicant claims the benefit of United Kingdom Application No. 9918788.2, filed August 10, 1999.

A certified copy of said application is appended hereto.

DATE: May 29, 2002

Respectfully submitted,

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EP 00/07874 4

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9918788.2

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)*

76055049001

If the applicant is a corporate body, give the country/state of its incorporation

BE

4. Title of the invention

Antithrombotic effect of platelet glycoprotein Ib blocking monoclonal antibody Fab fragments

5. Name of your agent *(if you have one)*

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ANTITHROMBOTIC EFFECT OF PLATELET GLYCOPROTEIN Ib BLOCKING MONOCLONAL ANTIBODY Fab FRAGMENTS .

ABSTRACT

Background: Platelet adhesion is mainly supported by the platelet receptor glycoprotein (GP) Ib, which binds to von Willebrand Factor (vWF). We have studied the in vivo effect of an inhibitory murine monoclonal anti-GPIb antibody (MoAb), 6B4, in a baboon model of arterial thrombosis.

Methods and Results: When injected into baboons, intact IgG1 and its $F(ab')_2$ fragments caused almost immediate thrombocytopenia, whereas injection of the Fab fragments did not. Fab fragments therefore were used to investigate their in vivo effect on platelet deposition on a thrombogenic device, consisting of gluteraldehyde fixed bovine pericardium (0.6 cm^2) at a wall shear rate ranging from 700 - 1,000 sec^{-1}. Baboons were either pretreated with Fabs to study the effect of inhibition on platelet adhesion, or treated 6 minutes after placement of the thrombogenic device, to investigate the effect on interplatelet cohesion. Pre-treatment of the animals with a bolus of 80 or 160 µg/kg Fab fragments reduced ^{111}In-labelled platelet deposition onto the collagen surface by approximately 52% and 61% respectively. Ex vivo ristocetin-induced platelet agglutination was equally reduced by approximately 40% and 50% respectively. Treatment with a bolus of 120 µg/kg Fab fragments after a thrombus was allowed to form for 6 minutes had no effect on further platelet deposition.

Conclusions: Whereas blockade of GPIb had no effect on platelet deposition onto a fresh thrombus, pretreatment effectively reduced thrombus formation.

DESCRIPTION

FIELD OF THE INVENTION

The present invention relates to novel cell lines and to ligands, namely human and/or humanized monoclonal antibodies, as well as fragments such as Fab or single variable domains and derivatives and combinations thereof, obtainable from the said cell lines. It also relates to pharmaceutical compositions comprising said ligands and to methods of preventing and treating coagulation disorders and in particular antithrombotic treatments in humans by administration of the said ligands to patients in need thereof.

In arterial blood flow the platelet adhesion is mainly supported by the platelet receptor glycoprotein (GP) Ib which interacts with von Willebrand factor (vWF) at the site of vessel wall injury. Blood platelets, through the processes of adhesion, activation, shape change, the release reaction and aggregation, form the first line of defence when blood vessels are damaged. They form a hemostatic plug at the site of injury to prevent excessive blood loss. Extensive platelet activation however may overcome the normal thromboregulatory mechanisms that limit the size of the hemostatic plug. The platelets then become major prothrombotic offenders predisposing to vaso-occlusive disease [1,2].

Platelet adhesion is regarded as the trigger for hemostasis and thrombosis. When subendothelial collagen is exposed, circulating vWF binds to it and, under the influence of high shear stress, undergoes a conformational change to bind to its receptor, GPIb/IX/V, on the platelet membrane [3]. This interaction is reversible and only slows down the progress of the platelets across the damaged surface [4]. Full immobilisation occurs when collagen binds to its receptor GPIa/IIa (integrin $\alpha_2\beta_1$) [5]. In addition, collagen activates platelets mainly by binding to GPVI, another collagen receptor [6,7]. When platelets are activated, GPIIb/IIIa (integrin $\alpha_{IIB}\beta_3$) undergoes a conformational change and acquires the ability to bind to fibrinogen and vWF which crosslinks adjacent platelets to form platelet aggregates.

Lately much effort has been directed to develop antibodies and

peptides that can block the binding of the adhesive proteins to GPIIb/IIIa and many of these are being tested in clinical trials [8-10]. On the other hand, the development of compounds that interfere with the vWF-GPIb axis has lagged behind. Only a few in vivo studies that investigated the effects of inhibition of platelet adhesion on thrombogenesis are described. They include the use of anti-vWF MoAbs [11,12], GPIb binding snake venom proteins like echicetin and crotalin [13,14], aurin tricarboxylic acid that binds to vWF [15] and recombinant vWF fragments like VCL [16], all of which inhibit vWF-GPIb interaction. All these molecules were antithrombotic, particularly in studies where a thrombus was formed under high shear conditions [12]. A number of potent inhibitory anti-GPIb antibodies have been produced and were extensively tested with respect to their in vitro effect under both static (platelet agglutination, vWF-binding) and flow conditions [17]. We are aware of only one successful in vivo study on guinea pigs where $F(ab')_2$ fragments of PG1, a monoclonal anti-guinea pig GPIb antibody, were used [18]. It was shown that it could effectively reduce the thrombus formation on a laser-induced injury. Unfortunately, this antibody does not cross react with human platelets. Part of this rather surprising lack of in vivo studies is due to the low cross reactivity of the anti-human GPIb MoAbs with platelets from commonly used laboratory animals. This predisposes to the use of non-human primates as experimental animals. However, even then attempts to perform in vivo studies are hampered because injection of the anti-GPIb MoAbs, as well as the snake venom protein echicetin that reacts with GPIb, invariably causes severe thrombocytopenia [11,13,18].

SUMMARY OF THE INVENTION

The present invention is summarised in the attached claims. The present invention includes a cell-line deposited with the Belgian Coordinated Collections of Micro-organisms, under accession number LMBP 5108CB from which monoclonal antibodies 64B may be obtained.

We studied the antithrombotic effect of platelet glycoprotein Ib blocking monoclonal antibody 6B4 Fab fragments derived from the antibodies from cell line LMBP 5108CB in a baboon model of arterial thrombosis. Baboons were

either pretreated with Fabs to study their effect on platelet deposition on a thrombogenic device, or treated 6 minutes after placement of the thrombogenic device, in order to investigate their effect on interplatelet cohesion. Blockade of GPIb had no effect on platelet deposition onto a fresh thrombus, whereas pretreatment effectively reduced thrombus formation.

We have studied the antithrombotic efficacy of the novel murine MoAb, 6B4 (IgG1), raised against human platelet glycoprotein Ib, in in vitro and in vivo studies. In vitro, 6B4 potently inhibits the binding of vWF to human GPIb both under static and flow conditions (unpublished results) and it also binds to baboon platelets. In the in vivo studies where 6B4 was injected into baboons, both the intact MoAb and its $F(ab')_2$ fragments caused immediate and severe thrombocytopenia, but the Fab fragments did not. Furthermore, Fab fragments studied in a baboon model of platelet-dependent arterial thrombosis inhibited thrombosis when the fragments were injected before a thrombus was generated in the baboons. On the other hand, when the Fab-fragments were injected after a thrombus was allowed to form, no inhibition of further thrombosis was observed.

The present invention also includes polynucleotide sequences which encode for the antibodies or fragments thereof mentioned above. It will be appreciated that a multitude of nucleotide sequences exist which fall under the scope of the present invention as a result of the redundancy in the genetic code. The present invention also includes complementary sequences which bind to the antibodies or fragments thereof mentioned above. In particular, the present invention includes probes constructed from the antibodies or fragments thereof mentioned above or from the polynucleotides or from the complementary sequences mentioned above.

LEGENDS TO FIGURES

Figure 1

Binding curves of anti-GPIb ^{125}I-6B4 IgG (■), - F(ab')2 (●) and - Fab fragments (▲) to baboon platelets in plasma. Data are mean of duplicate

measurements in PRP from two baboons.

Figure 2

Effect of anti-GPIb 6B4 IgG (■), - F(ab')2 (●) and - Fab fragments (▲) on ristocetin-induced baboon platelet aggregation. Data, expressed as % inhibition, are mean of duplicate measurements in PRP from two baboons.

Figure 3

Platelet deposition onto three thrombogenic devices, containing bovine pericardium, placed consecutively at times 0 (●), 60 (■) and 120 (▲) minutes for 30 minutes (top shaded bars). Panel A: Sham experiments (n=4). Panel B: Following injection of 0 (●), 80 (■) and 160 (▲) µg/kg 6B4 Fab fragments (n=5). Values are given as mean ± SE.

Figure 4

Influence of late treatment of baboons with 6B4 Fab fragments on platelet deposition. The thrombogenic device was placed at time 0 and platelet deposition determined for 24 minutes (top shaded bar). After six minutes (arrow) animals (n=6) were either treated with 0 (■) or a bolus of 110 (●) µg/kg 6B4 Fab fragments. Values are given as mean ± SE.

METHODS

Preparation and purification of intact MoAb 6B4, F(ab')$_2$ and Fab fragments

The antibody used in these studies, 6B4 (subtype IgG1), is a murine monoclonal antibody (MoAb) raised against purified human GPIb and obtainable from the cell line having the accession number LMBP 5108CB. When added at saturating concentrations, MoAb 6B4 totally abolishes both ristocetin- and botrocetin-induced human platelet aggregation as well as shear-induced platelet adhesion to human collagen type I tested in a Sakariassen-type flow chamber at 2600s^{-1}.

Hybridoma cells producing the MoAb 6B4 were grown and subsequently injected into pristane-primed Balb/c mice. After 10 days ascites fluid was collected. The IgG was extracted from the ascites using protein-A-Sepharose CL-4B (Pharmacia, Roosendaal, The Netherlands).

To prepare $F(ab')_2$ fragments, MoAb 6B4 was dialyzed overnight against a 0.1mol/L citrate buffer (pH 3.5) The antibody was digested by incubation with pepsin (Sigma,St Louis, MO; 1 part pepsin to 200 parts MoAb) for 1 hour at 37°C. Digestion was stopped by adding 1 volume of a 1M TrisHCl buffer (pH 9) to 10 volumes of antibody.

Monovalent Fab fragments were prepared by papain digestion. A 1:10 (vol:vol) of 1M phosphate buffer (pH 7.3) was added to the antibody. Papain (Sigma) was added at a ratio of 1 vol papain to 25 volumes of the phosphate buffer containing the MoAb, 10mmol/L L-Cysteine HCl (Sigma) and 15mmol/L EDTA. After incubation for 3 hours at 37°C, digestion was stopped by adding a final concentration of 30mmol/L freshly prepared iodoacetamide solution (Sigma), keeping the mixture in the dark at room temperature for 30 minutes.

Both $F(ab')_2$ and Fab fragments were further purified from contaminating intact IgG and Fc fragments using protein-A-Sepharose. The purified fragments were finally dialyzed against PBS. Purity of the fragments was determined by SDS-PAGE and the protein concentration was measured using the BCA Protein Assay Reagent A (Pierce, Rockford, IL).

Animal studies

Normal male baboons (*Papio ursinus*) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks prior to use. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and

Testing of Drugs and Related Substances in South Africa.

The baboons supported permanent Teflon-Silastic Arteriovenous (AV) shunts implanted in the femoral vessels [20]. Blood flow through the shunts varied between 100 and 120 mL/min, resulting in wall shear rates between 800 and 1,000 sec^{-1}, which compares with the shear rates found in medium sized arteries [20].

Handling of the baboons was achieved through anaesthesia with ketamine hydrochloride (~10mg/kg, i.m.) (Anaket-V, Centaur Laboratory, South Africa).

Study protocol

In order to test the effect of the MoAb on platelet count, 6B4 and its $F(ab')_2$ and Fab fragments were administered to three different baboons. The injected dose was calculated to attain a plasma concentration of $1xKD_{50}$ i.e. the concentration needed to occupy 50% of the receptors as determined in in vitro experiments (see further).

Platelet-dependent arterial thrombus formation was induced by using bovine pericardium (0.6 cm^2) fixed in buffered gluteraldehyde [21]. The pericardium was built into the wall of silicone rubber tubing (3 mm inside diameter). The method of preparation of the thrombogenic device is described in detail [22], except that fixed bovine pericardium instead of Dacron vascular graft material was used. In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV-shunt by means of teflon connectors [20].

Two approaches were followed to determine the effect of 6B4 Fab fragments on platelet adhesion and interplatelet cohesion onto the collagen of the bovine pericardium:

Dose response effect of 6B4 Fab-fragments on platelet adhesion and deposition: Nine baboons were used in the first studies. In five baboons, a thrombogenic device was placed to determine deposition of platelets (see

Graft imaging and quantification of platelet deposition). After 30 minutes, the device was removed and blood flow through the permanent AV-shunt re-established. The baboon was then treated with a bolus of Fab fragments of 6B4 (in 2 ml saline) aimed to attain a plasma concentration of approximately 0.75 x KD_{50}. Thirty minutes after treatment, a second thrombogenic device was placed for 30 minutes to determine the effect of the Fab fragments on thrombogenesis. The device was again removed and blood flow through the permanent shunt established. This was followed by bolus injection of Fab fragments to attain a cumulative plasma concentration of approximately 1.5 x KD_{50}. After 30 minutes, a third thrombogenic device was placed for 30 minutes and platelet deposition measured. The dose of Fab fragments required to attain the chosen plasma concentration was calculated, based on the dose response curve obtained in vitro (see receptor binding studies). Briefly, the plasma volume was calculated by assuming a blood volume of 65ml/kg body mass and correcting for the haematocrit [22].

Sham studies were done in four other baboons. In these studies, the same protocol of placement of thrombogenic devices was followed, but the baboons were not treated with Fab fragments.

Effect of anti-GPIb 6B4 fragments on interplatelet cohesion: In a second series of studies 12 baboons were used. In all baboons, a thrombogenic device was placed for 24 minutes. Six received a bolus injection of Fab fragments to attain a plasma concentration of approximately 1.0 x KD_{50}. The fragments were injected six minutes after placement of the thrombogenic device to allow enough platelets to be deposited to cover the collagen surface. The six other baboons did not receive Fab fragments.

In both approaches, blood was collected at different time points to determine platelet count and haematocrit (EDTA), circulating and platelet associated radioactivity, the *ex vivo* aggregation of platelets in response to ristocetin and the plasma concentrations of Fab fragments (see Laboratory measurements).

The time points at which the blood was collected are given in the figures.

Graft imaging and quantification of platelet deposition

Autologous blood platelets were labelled with ^{111}In-tropolone as previously described [23]. Imaging and quantification of the deposition of ^{111}In-platelets were done as described in detail [22,23]. Briefly, image acquisition of the grafts, including proximal and distal silastic segments, was done with a Large Field of View scintillation camera fitted with a high resolution collimator. The images were stored on and analysed with a Medical Data Systems A^3 computer (Medtronic. Ann Arbor. MI) interfaced with the scintillation camera. Dynamic image acquisition, 2 minute images (128x128 byte mode), was started simultaneously with the start of blood flow through the devices. A two minute image (128x128 byte mode) of a 3 ml autologous blood sample (collected in EDTA) was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined, and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition was expressed as the total number of platelets deposited. The method to calculate this is described in detail [20].

Laboratory measurements

Receptor binding studies: 6B4, its F(ab')$_2$ or Fab fragments were labelled with Na-^{125}I (Amersham, Buckinghamshire, UK) using the Iodogen method [24]. Iodogen was purchased from Pierce (Rockford, IL). Platelet-rich baboon plasma, adjusted with autologous plasma to a count of 100,000 platelets/µl, was incubated with different concentrations of iodinated 6B4, F(ab')2 or Fab

fragments for 15 minutes at room temperature. The mixture was layered onto 20% sucrose buffer (wt/vol) containing 0.1% (wt/vol) bovine serum albumin (BSA) and centrifuged for 4 min at 10,000g in Eppendorf tubes. The top fluid, including the plasma, was removed and the pellets were counted in a gamma-counter. Binding studies were performed in duplicate on the PRP of two baboons.

In vitro and ex vivo platelet aggregation: The aggregation of platelets in response to ristocetin (1.5mg/mL final concentration; abp, NY) was done on 10 ml blood collected in 1 ml of 3.2% trisodiumcitrate. Platelet rich plasma (PRP) was prepared by differential centrifugation [25] and the platelet count adjusted to 200,000 platelets/µl with autologous plasma. The aggregation response was measured in a Monitor IV Plus aggregometer (Helena Laboratories, Beaumont, TX) and recorded for 5 minutes. The percent aggregation at 5 minutes was calculated as the difference in light transmission between platelet-rich and platelet-poor plasma.

In *in vitro* studies, the PRP was preincubated for 5 minutes with serial dilutions of intact IgG 6B4, F(ab')2 or Fab fragments before aggregation was initiated. Inhibition of aggregation was calculated from the difference in the aggregation response of platelets without and with antibody or fragments. In the *ex vivo* determinations, inhibition was calculated from the difference in the aggregation response of platelets before and after treatment of the baboons.

Plasma levels of 6B4, F(ab')$_2$ or Fab fragments: were measured using a sandwich ELISA. Briefly, microtiter plates were coated overnight at 4°C with 5µg/mL polyclonal goat anti-mouse IgG (Sigma). After blocking non-occupied binding sites with bovine serum albumin, serial dilutions of baboon plasma were added to the wells and incubated for two hours. Bound 6B4 (IgG, F(ab')$_2$ or Fab fragments) was detected by using goat anti-mouse IgG (Fab specific) conjugated to peroxidase (Sigma). Standard curves were constructed by adding known amounts of 6B4 (IgG, F(ab')$_2$ or Fab fragments) to baboon

plasma.

Bleeding time: The bleeding time was determined using the Simplate® II device (Organon Teknika,Durham, NC) according to the instructions of the manufacturers. The volar surface of the forearm of the baboons was shaved, a pressure cuff was applied and inflated to 40 mm Hg.

Statistical analysis

Student's t-test for paired data was used to test for statistically significant difference. Data given in the text are mean ± SE. P-values < 0.05 are considered significantly different.

RESULTS

In vitro effect of MoAb 6B4 and its F(ab')$_2$ and Fab fragments on baboon platelets

Binding of the antibody and its fragments to baboon platelets was dose-dependent and saturable. Half saturation (KD_{50}) was obtained with 4.7 nmol/L, 6.4 nmol/L and 49.2 nmol/L for 6B4 IgG, F(ab')$_2$ and Fab fragments respectively (Fig1). When added at saturating concentrations, ristocetin-induced aggregation was completely abolished (Fig 2). IC_{50}-values were 4.5 nmol/L, 7.7 nmol/L and 40 nmol/L for 6B4 IgG, F(ab')$_2$ and Fab fragments respectively.

Effect of injection of MoAb 6B4, F(ab')$_2$ and Fab fragments on the peripheral platelet count in baboons

For purposes of comparison, the dose of the MoAb 6B4 and its fragments used were calculated to attain a plasma concentration of $1xKD_{50}$. In one baboon, the intact antibody caused a profound decrease in the blood platelet

count (< $30x10^8$pl/L) within 10 minutes after injection. After 48 hours, the platelet count was still below $100x10^9$pl/L. When 6B4 $F(ab')_2$ fragments were injected into 2 baboons, the platelet count decreased rapidly to between 120 and $150x10^9$pl/L, i.e. by approximately 60% and then reached pre-infusion values within 24 hours. When the monovalent 6B4 Fab fragments were injected. the platelet count decreased by less than 10%. On the basis of this, the 6B4 Fab fragments were used for further studies.

Effect of different doses of MoAb 6B4 Fab fragments on platelet deposition

Platelet adhesion and deposition onto thrombogenic devices sequentially placed 30 minutes apart, is summarized in Figure 3. In the sham studies (Fig 3A), placement of the previous graft had no significant effect on platelet deposition on subsequent grafts. The first two grafts that were placed also occluded before the end of 30 minutes as visualized by arrest of blood flow through the tubing. In the treatment studies (Fig 3B) dosages to attain plasma concentrations of $0.75xKd_{50}$ (80 µg/kg) and $1.5xKd_{50}$ (160 µg/kg) significantly inhibited platelet deposition by approximately 52% and 61% respectively. The difference in inhibition by the different dosages was not significant.

Plasma levels of 6B4 Fab-fragments and inhibition of ex vivo agglutination determined on samples obtained 30 minutes or 2 hours after administration both were dose and time dependent (Table 1).

Bleeding times, determined in the treatment studies before and 30 minutes after injection of the 6B4 Fab fragments, prolonged from 2.6±0.3 to 5.4±1.2 min, which was not significant.

Effect of 6B4 Fab fragments on interplatelet cohesion

Treatment of the baboons with a dose to attain a plasma concentration of 1.0 x KD_{50} did not affect platelet deposition when it was injected after a thrombus

was allowed to form for 6 minutes (Figure 4).

DISCUSSION

The anti-GPIb MoAb 6B4, its $F(ab')_2$ and Fab fragments prevent the binding of vWF to human GPIb and hence ristocetin-induced human platelet agglutination. They also bind to and inhibit baboon platelets in vitro. An almost immediate and profound thrombocytopenia followed when the intact antibody was injected into baboons, similar to what was seen when other anti-GPIb MoAbs were injected into different experimental animals [11,18].

Thrombocytopenia lasted for up to 48 hours, which strongly suggests that the platelets were permanently removed from the circulation. Injection of the 6B4 $F(ab')_2$ fraction likewise caused immediate thrombocytopenia but to a lesser extent than the intact antibody. Unlike with the intact antibody, thrombocytopenia was reversible since the platelet count returned to normal within 24 hours. The differential results obtained with the intact IgG and F(ab')2 fragments indicate that the thrombocytopenic effect is to a large extent dependent on the Fc portion of the antibody.

Another observation, as evidenced by electron microscopy studies, was that both the intact IgG 6B4 as well as F(ab')2 fragments induced the formation of platelet agglutinates, when added to baboon platelet rich plasma at saturation concentrations and stirred (data not shown). Platelet agglutinates therefore can be formed as a result of platelet bridging through the antigen-binding sites of the antibody. Such agglutinates were not observed when adding the monovalent Fab fragments. Injection of the 6B4 Fab fraction caused only a mild and negligible decrease (<10%) of the blood platelet count. It can therefore be hypothesized that the thrombocytopenia induced by both bivalent Ab, is not only caused by the Fc-part of the Ab but may also occur through the formation of platelet agglutinates that are cleared from the circulation.

The platelet antigen can also be important to determine whether thrombocytopenia will occur. GPIb may be a particularly sensitive receptor, since its highly glycosylated mucin-like area keeps the receptor in an extended, rod like conformation [26]. As a result, the vWF and thrombin binding domains are exposed far above the platelet glycocalix. Since inhibitory antibodies are expected to bind to the same domain, they may be optimally presented for clearance by Ab recognising systems. Indeed, patients with autoantibodies against GPIb most often develop severe immune thrombocytopenia [27].

In view of the thrombocytopenia induced by both IgG and F(ab')2 fragments, we decided to assess the antithrombotic effect of the Fab-fraction in a baboon model of arterial thrombosis [20]. The gluteraldehyde fixed bovine pericardium was highly thrombogenic: after 30 minutes of exposure to native flowing blood, approximately $3x10^9$ platelets deposited on the area of 0.6 cm^2. In similar studies, Dacron vascular graft material (0.9 cm^2) only accumulated approximately $0.7x\ 10^9$ platelets in 30 minutes [22]. It is therefore also not surprising that the control thrombogenic devices occluded before 30 minutes of exposure to flowing blood.

Treatment of baboons with 6B4 Fab-fragments inhibited platelet deposition on the thrombogenic devices by between 50 and 60%, and prevented occlusion of the thrombogenic devices. The observed effect must be ascribed to the effect of the antibody, since sequential placement of thrombogenic devices in untreated baboons caused no decreased deposition. The inhibitory effect of the Fab fragments on platelet deposition paralleled changes in ex vivo ristocetin-induced platelet agglutination tests: a bolus of 80µg/kg resulted in 52% and 39% inhibition of deposition and agglutination respectively, a cumulative dose of 160µg/kg gave 61% and 54% inhibition. The increased inhibition caused by the higher concentrations of Fab fragments however was not statistically significant. The doses we used caused a significant reduction in thrombus size and prevented the formation of an occluding thrombus

without increasing the risk of bleeding since the bleeding time was not significantly prolonged.

On the other hand, it is also possible that the shear conditions we used can only result in a maximum of 60% inhibition of the collagen-vWF-GPIb axis, similar to that obtained with an anti-vWF MoAb BB3BD5 [11]. The inhibitory effect could therefore be more pronounced at higher shear. In addition direct platelet-collagen interactions also contribute to the platelet deposition, relative to the contribution of the GPIb-vWF axis [5-7].

Since binding of vWF to GPIb also activates platelets [28,29], it is reasonable to assume that less platelets will be activated, or that a lower level of platelet activation will be achieved when binding is inhibited. The smaller thrombus that finally forms may therefore be a consequence of both fewer platelets that adhere to the collagen and less platelet activation. In addition, the GPIb/IX/V complex may also be involved in platelet-platelet interaction [3,4]. To test this we did a second series of studies to investigate the role of GPIb/IX/V complex in platelet-platelet interactions at intermediate shear rates. A thrombogenic device was placed as an extension segment in the permanent AV shunt and exposed to native flowing blood. After six minutes the baboons were treated with the Fab fractions of 6B4. We argued that six minutes exposure (number of platelets deposited approximately $0.6x10^9$) was sufficient to allow ample coverage of the pericardium with platelets. Inhibition of platelet deposition due to treatment, when compared with sham studies would therefore reflect inhibition of platelet-platelet interactions. Since no such effect was seen, this strongly suggests that the GPIb/IX/V complex does not play a major role in platelet-platelet interactions in vivo under the conditions studied. To our knowledge, these are the first in vivo results to show that GPIb has no influence on platelet-platelet interactions, and may help to clarify the role of GPIb in thrombogenesis [4].

In conclusion, our studies confirm the predominant role of GPIb in platelet

adhesion as compared to platelet aggregation, also in vivo. The present findings furthermore indicate that interfering with the collagen-vWF-GPIb axis is especially interesting to prevent, rather than to interrupt, arterial thrombus formation.

The formation of a platelet plug during primary hemostasis and of an occluding thrombus in arterial thrombosis involves common pathways. The first event is platelet attachment to the site of vessel injury, which can be a ruptured atherosclerotic plaque. Adhesion of platelets under high shear rate depends on the function of glycoprotein Ib (GP Ib) that mediates attachment of platelets to von Willebrand factor (vWF) multimers bound at the site of the exposed lesion. The GPIb-vWF interaction thus is an absolute necessity in order to produce a thrombus, at least in smaller vessels or stenosed arteries where shear-stress is high[30,31].

After the initial adhesion platelets will aggregate, an interaction that is assured through the binding of glycoprotein IIb-IIIa complexes with multivalent ligands, in particular fibrinogen and vWF[32]. A number of MoAbs has been developed that bind to GPIIb/IIIa and block its ability to bind fibrinogen[33,34]. Many investigators have shown that GPIIb-IIIa antagonists are antithrombotic in vivo[35,36]. Fab fragments of an anti-GPIIb/IIIa monoclonal antibody (7E3) were prepared in a chimeric humanized form (RheoPro) and tested in several clinical studies. Use of it has been found to be succesful in inhibiting acute thrombotic events after the physical expansion of a stenotic vessel by a balloon catheter (PTCA)[37,38]. Moreover nowadays RheoPro is approved for use in patients undergoing high-risk coronary artery angioplasty and atherectomy. However the major disadvantage in the clinical application of RheoPro, is its high bleeding risk in case of excessive inhibition of platelet aggregation[39].

Therefore the present invention includes an additional protective role by poviding an inhibitor of platelet adhesion that antagonizes platelet

glycoprotein Ib receptors without inducing a risk of bleeding and/or with which other advantages can be expected.

The main core of our observations is that by using monovalent Fab fragments of an inhibitory GPIb antibody, a profound prevention of platelet dependent thrombus formation can be obtained, without thrombocytopenia. Although a number of potent inhibitory anti-GPIb antibodies have been produced that block the binding with vWF, so far no other anti-human GPIb MoAb/fragments has been succesfully studied in vivo. Part of this rather suprising lack of in vivo studies with other anti-GPIb MoAbs is due to the low cross-reactivity of the anti-human GPIb MoAbs with platelets from commonly used laboratory animals. However even then attemps to perform in vivo studies are hampered because injection of the anti-GPIb MoAbs invariably causes severe thrombocytopenia[40,41]. Only two in vivo studies are reported where F(ab')2 fragments of an anti-guinea pig GPIb MoAb PG-1 were evaluated. The F(ab')2 fragments of this antibody significantly prolonged the occlusion time to arterial microvascular graft thrombosis without prolonging the bleeding time[42]. In another guinea-pig model, the fragments could effectively reduce the thrombus formation on a laser-induced injury[43]. However this antibody is specific for guinea-pig platelets and does not cross-react with human platelets. Secondly, animals treated with this divalent fragments developed mild thrombocytopenia.

The present invention includes antithrombotic therapies based on inhibition of the GPIb-vWF axis, as shown by our study. Few other compounds interfering at this level have been evaluated. In the following paragraph we will shortly review the antithrombotic efficacy of these compounds and state why, until now, they have not been considered worthwhile for further antithrombotic development.

The recombinant vWF fragment VCL, that binds to GPIb and inhibits platelet-vWF interaction[44], was found to be antithrombotic in several animal models. It

abolishes cyclic flow variations in stenosed coronary arteries of non-human primates[45], delays coronary artery reocclusion in a canine-model of tissue-type plasminogen activator-induced thrombolysis[46] and inhibits intimal thickening after balloon injury in the rat[47]. Moreover, the investigators report that VCL has only a minor effect on bleeding tendency. In order to be effective, high concentrations VCL (3-4mg/kg) are needed. Moreover, this peptide has only a short half-life of 20-30 minutes following intravenous administration[45,46]. Since 1997 we found no publications where it has been evaluated in new/other thrombosis models.

The MoAb AJvW-2 is a murine anti-human vWF antibody that abolishes GPIb-vWF interactions in various species. This antibody was shown to inhibit both injury induced arterial and venous thrombosis in hamster[48], and to prevent arterial thrombus formation in guinea-pigs[49]; again without an enhanced risk of bleeding at doses that were antithrombotic. However in this case the ligand vWF is blocked instead of its receptor GPIb. VWF is present in plasma and subendothelium, and additionally can be secreted by platelets after activation. Therefore an efficient vWF blocker should neutralize both subendothelial and plasma vWF. Also, in vitro studies have shown that the adhesion of blood platelets to the extracellular matrix of cultured human endothelial cells can be fully blocked by an anti-GPIb MoAb whereas an anti-vWF MoAb, blocking the vWF interaction with platelets, has a less pronounced effect. This indicates that GPIb may also have another role in platelet adhesion, apart from serving as the binding site for vWF[50].

The snake venom proteins echicetin and crotalin block vWF binding to GPIb. Both have been tested in mice and echicetin was found to induce a transient thrombocytopenia[51], crotalin does not. Crotalin could delay platelet-rich thrombus formation in mesenteric microvessels in a mouse model[52]. However at the effective dose it caused a significant prolongation of the bleeding time. Secondly, as suggested by the investigators, crotalin is a less effective blocker of the GPIb-vWF interaction in humans.

The organic compound aurintricarboxylic acid (ATA), that binds vWF high multimers and subsequently blocks GPIb-vWF interaction, has been tested in arterial thrombosis models in several animals[53,54]. The major disadvantage is that, at the effective dose, it prolongs the prothrombin time (PT), the activated partial thromboplastin time (APTT) and bleeding time.

All these studies thus show that blocking GPIb-vWF interaction can be an attractive tool for anti-thrombotic therapy, however until now no useful product is found. Therefore inhibition of GPIb-VWF axis through Fab fragments of an anti-GPIb MoAb is an attractive route for the prevention and treatment of acute coronary artery syndromes; and provides a new class of antithrombotic compounds.

Unfortunately, murine Abs are highly immunogenic in humans, limiting their potential use for human therapy especially when repeated administration is necessary. To reduce the immunogenicity of the murine anti-GPIb MoAb 6B4, the present invention includes construction of a chimeric Ab, preferentially as a scFv, which combines the variable region of the mouse Ab with a human Ab constant region - a so-called humanized monoclonal antibody. The monoclonal antibodies produced in animals may be humanized, for instance by associating the binding complementarity determining region ("CDR") from the non-human monoclonal antibody with human framework regions - in particular the constant C region of human gene - such as disclosed by Jones et al. in *Nature* (1986) 321:522 or Riechmann in *Nature* (1988) 332:323.

The major advantage of an antithrombotic product blocking the GPIb-vWF axis is its minor effect on bleeding events (shown by our study and studies with VCL[45,46], AJvW-2[48]). Until now, the high bleeding risk forms the major disadvantage in the clinical application of GPIIb/IIIa antagonists. Apart from this, other advantages can justify the effort in the development of a GPIb-blocker.

The GPIb-vWF interaction is the ultimate first step in platelet adhesion. This interaction leads to platelet activation[55] and engagement of other receptors that will get involved in the thrombus growth. The GPIb-vWF axis therefore presents an attractive alternative to GPIIb/IIIa-fibrinogen as a target for platelet inhibition, since a suitable inhibitor might be expected to down regulate other manifestations of platelet activity such as granule release, thought to play a role in the development of arteriosclerosis.

Activation of platelets is accompanied by secretion of vasoactive substances (thromboxane A2, serotonin) as well as growth factors such as PGDF[58]. Therefore, early inhibition of platelet activation and hence prevention of the secretion of their growth and migration factors, via a GPIb blocker, would reduce the proliferation of smooth muscle cells and restenosis after thrombolytic therapy. In this aspect ATA[57] and VCL[47] have been shown to limit restenosis.

The beneficial effect of RheoPro on restenosis to a large extent seem to be due to its cross-reactivity with smooth muscle cell $\alpha_v\beta_3$ (α_vGPIIIa)[37]. Newer (orally available) peptidomimetics tend to be more specific but may have little if any effect on restenosis. Again, where GPIIb-IIIa blockers mainly prevent platelet aggregation, interruption at an earlier stage by a GPIb blocker can maybe not only limit the platelet plug that is formed, but also reduce additional platelet dependent effects.

We believe that the use of a GPIb blocker will probably be more useful in acute situations and as adjunctive therapy with other agents (aspirin and heparin). The pharmaceutical composition of the present invention may further comprise, in view of the so-called adjunctive anti-thrombotic treatment, a therapeutically effective amount of a thrombolytic agent. Such thrombolytic agents, as well as their usual dosage depending on the class to which they belong, are well known to those skilled in the art. Among numerous examples

of thrombolytic agents which may be included in the pharmaceutical compositions of the invention, the following non-limiting list may be particularly cited: T-Pa, streptokinase, reptilase, TNK-t-Pa or staphylokinase.

Suitable pharmaceutical carriers for use in the pharmaceutical compositions of the invention are described for instance in Remington's Pharmaceutical Sciences 16th ed. (1980) and their formulation is well known to those skilled in the art. They include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like. Additional ingredients may be included in order to control the duration of action of the monoclonal antibody or FAB fragment active ingredient in the composition. Control release compositions may thus be achieved by selecting appropriate polymer carriers such as for example polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinyl acetate copolymers, methylcellulose, carboxymethylcellulose, protamine sulfate and the like. The rate of drug release and duration of action may also be controlled by incorporating the monoclonal antibody active or FAB fragment ingredient into particles, e.g. microcapsules, of a polymeric substance such as hydrogels, polylactic acid, hydroxymethylcellulose, polymethyl methacrylate and the other above-described polymers. Such methods include colloid drug delivery systems like liposomes, microspheres, microemulsions, nanoparticles, nanocapsules and so on. Depending on the route of administration, the pharmaceutical composition comprising the active ingredient may require protective coatings.

The pharmaceutical form suitable for injectionable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation thereof. Typical carriers therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol and mixtures thereof.

Ligands in accordance with the present invention, e.g. Fab fragments of the cell line mentioned above, may be provided to a patient by means well known

in the art. i.e. orally, intranasally, subcutaneously, intramuscularly, intradermally, intravenously, intraarterially, parenterally or by catheterization. According to the present invention, the ligand may be used as a medicament in conjunction or association with a thrombolytic agent such as disclosed hereinabove under the heading of pharmaceutical compositions.

Of course, apsirin will continue to be widely used for patients with vascular disease[58]; however there are a number of situations in which increased thrombotic risk requires the use of a more potent platelet inhibitor than aspirin[59]. Conditions such as angioplasty, coronary stenting and thrombolysis are likely to require more potent platelet inhibitors. In these acute clinical situations, the fibrous cap over an atherosclerotic plaque has been ruptured which produces deep arterial injury and exposes a much more thrombogenic surface. Furthermore, high shear forces acting on platelets passing through severely narrowed stenoses can also overcome the inhibitory effects of aspirin[60].

Therefore a GPIb antagonist may be used for reducing the problems of occlusion and restenosis in patients undergoing angioplasty or for the prevention of reocclusion after succesful thrombolysis by t-PA, streptokinase. It is believed that platelet activation, as a result of the platelet adhesion, is a key component in the failure of thrombolysis. Therefore a therapeutic approach towards blocking the GPIb-vWF interaction, that would result in a down-regulation of platelet signalling, represents a new way of interfere in thrombus formation.

Again, in these acute situations also GPIIb-IIIa antagonists have been shown to be effective. However we believe that there is an additional protective role for an inhibitor of platelet adhesion, by antagonizing the GPIb receptor, with a lower risk of bleeding and additional advantages resulting from the early intervention of platelet activation.

Table 1.

Plasma levels of 6B4 Fab-fragments and ex vivo ristocetin-induced platelet agglutination, upon administration of 0.75 and 1.5 x KD_{50} (A) to baboons. Values are given as a mean ± SE.

Dose (µg/kg)	Time (min)	Plasma levels (µg/mL)	% Inhibition of ex vivo ristocetin-induced platelet agglutination
0	Pre	0	0
80	90	0.671 ± 0.046	39 ± 9.9
160	150	1.053 ± 0.091	54 ± 7.2
	270	0.475 ± 0.031	46 ± 4.1

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CLAIMS

1. Cell line deposited with the Belgian Coordinated Collections of Micro-organisms. under accession number LMBP 5108CB.

2. Cell line producing monoclonal antibodies having a reactivity substantially identical to that of the monoclonal antibodies obtained from the cell line of claim 1.

3. A ligand which binds to the human platelet glycoprotein GP1b and prevents the binding of vWF to GP1b.

4. A ligand, in particular according to claim 3, being derived from a monoclonal antibody obtainable from the cell line of claim 1 or claim 2.

5. The ligand according to claim 4, being a FAB fragment of the monoclonal antibody.

6. The ligand according to claim 4 or 5, being able to recognize an epitope located on human GP1b.

7. The ligand according to any of claims 3 to 5 and being derived from a monoclonal antibody produced by on purpose immunization in animals, preferably in a mouse.

8. A humanized monoclonal antibody derivable from the monoclonal antibody of claim 7 or derivable from the cell lines of claims 1 or 2.

9. An antigen-binding Fab fragment or a homolog or derivative thereof of a monoclonal antibody according to claims 7 or 8 or derived from the cell lines of claims 1 or 2.

10. A pharmaceutical composition for the prevention or treatment of disorders of hemostasis in humans, particularly for antithrombotic use, comprising a ligand according to any of claims 3 to 7. a humanized monoclonal antibody according to claim 8 or an antigen binding Fab fragment according to claim 9, in admixture with a pharmaceutically acceptable

carrier.

11. A pharmaceutical composition according to claim 10, further comprising a thrombolytic agent in a form either for simultaneous or sequential use.

12. A method of treatment and/or prevention of a disorder of hemostasis, a coagulation disorder or attenuation of coagulation in a human, particularly for antithrombotic use, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a ligand according to any of the claims 3 to 7, a humanized monoclonal antibody according to claim 8, or an antigen binding Fab fragment according to claim 9.

13. A method according to claim 12, wherein the ligand or Fab fragment or homolog thereof is provided to a patient by oral, intranasal, subcutaneous, intramuscular. intradermal, intravenous, intraarterial or parenteral administration or by catheterization.

14. A method according to claim 12 or 13, further comprising sequentially or simultaneously administrating to the patient a therapeutically effective amount of a thrombolytic agent.

15. A polynucleotide encoding for an antigen-binding Fab fragment according to claim 9.

Fig 1

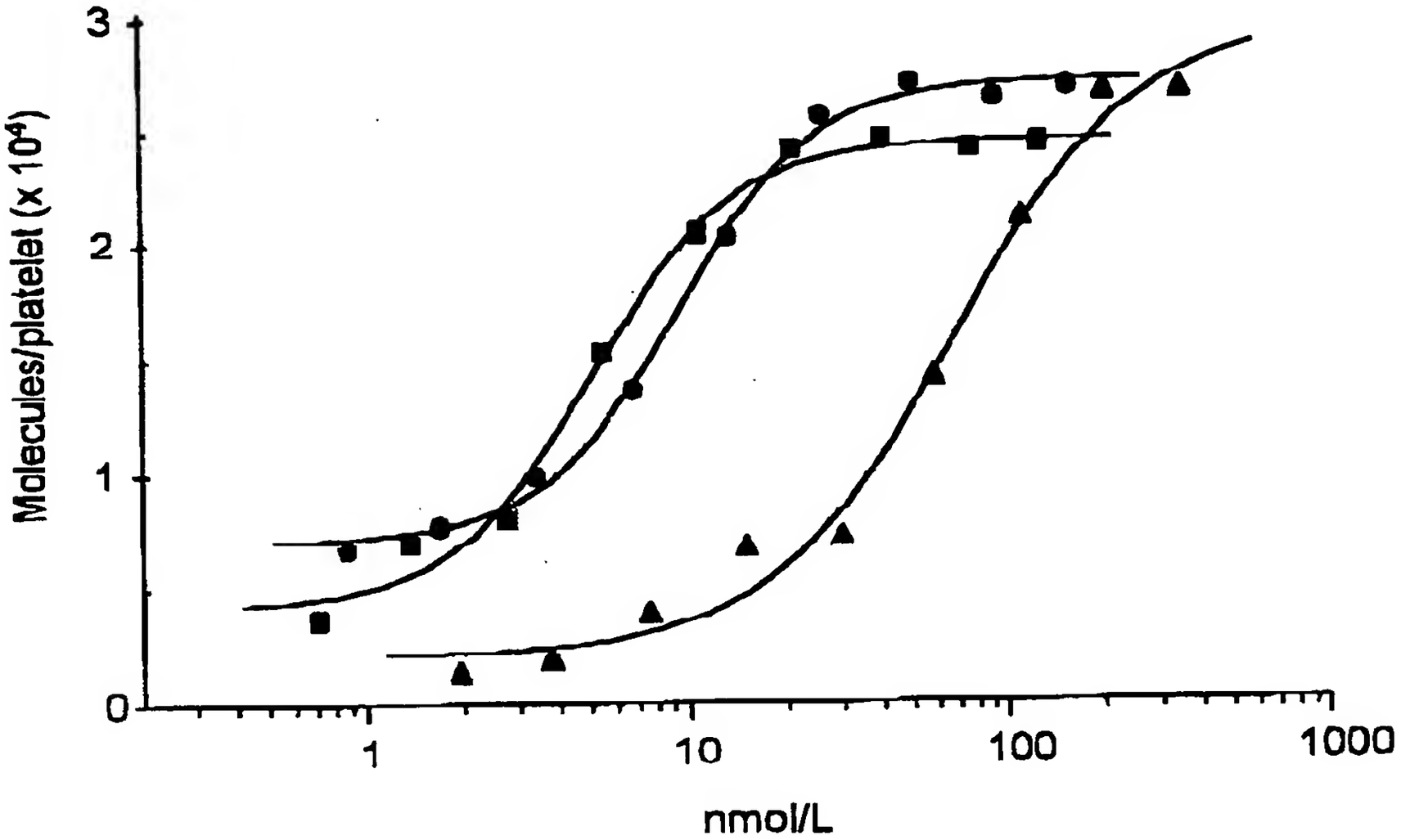


Fig 2

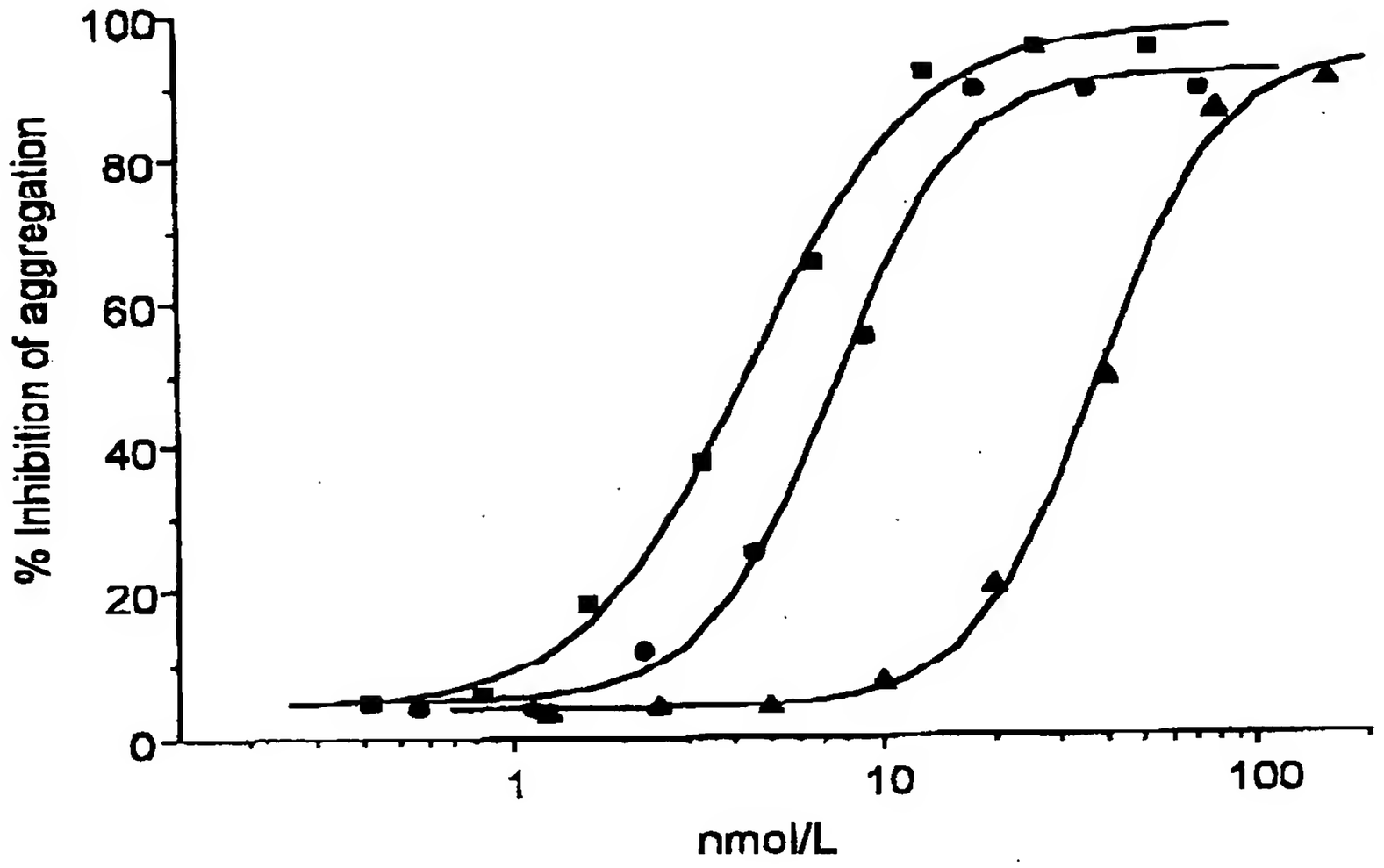
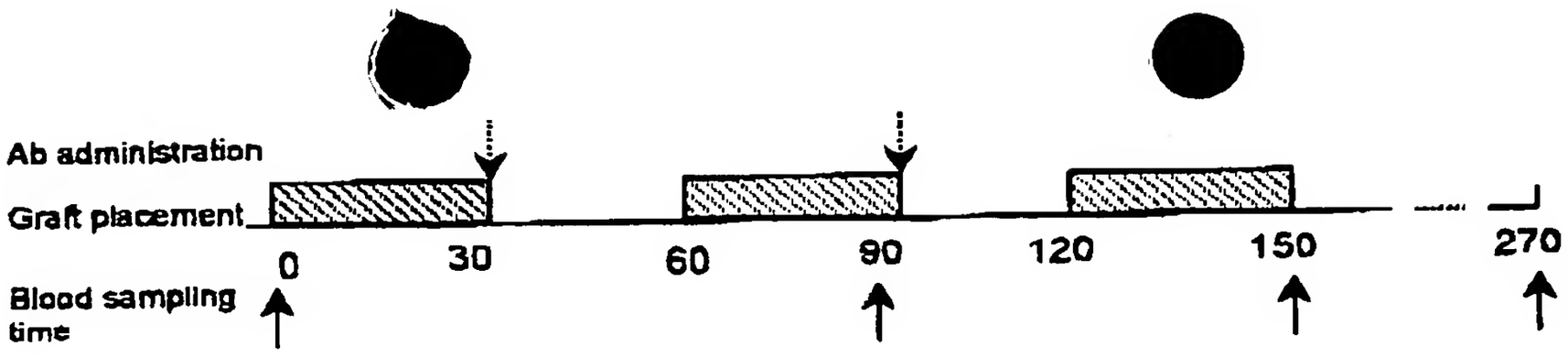
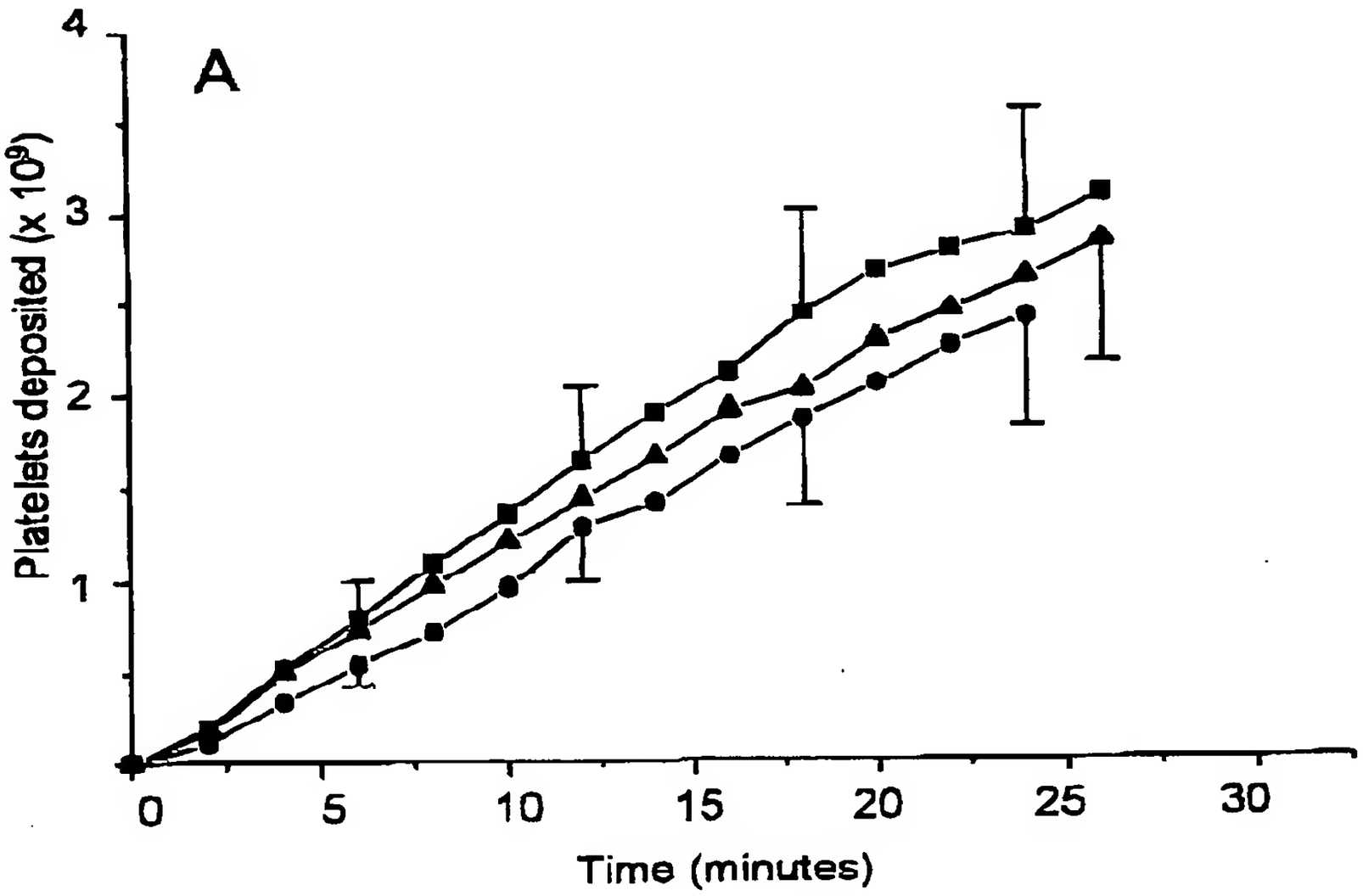


Fig 3


Ab administration
Graft placement
Blood sampling time
0
30
60
90
120
150
270


A
Platelets deposited (x 10⁹)
1
2
3
4
0
5
10
15
20
25
30
Time (minutes)

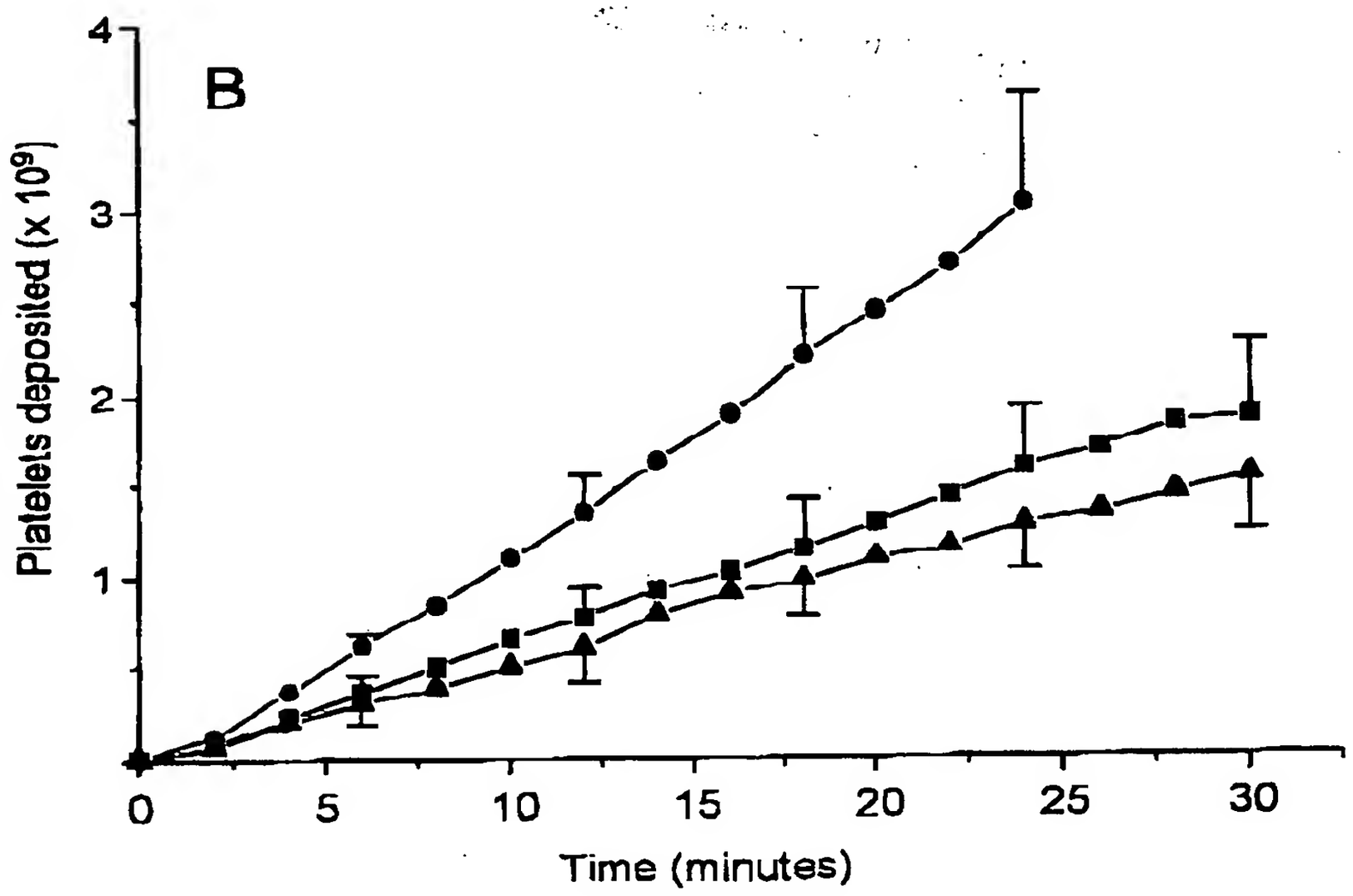
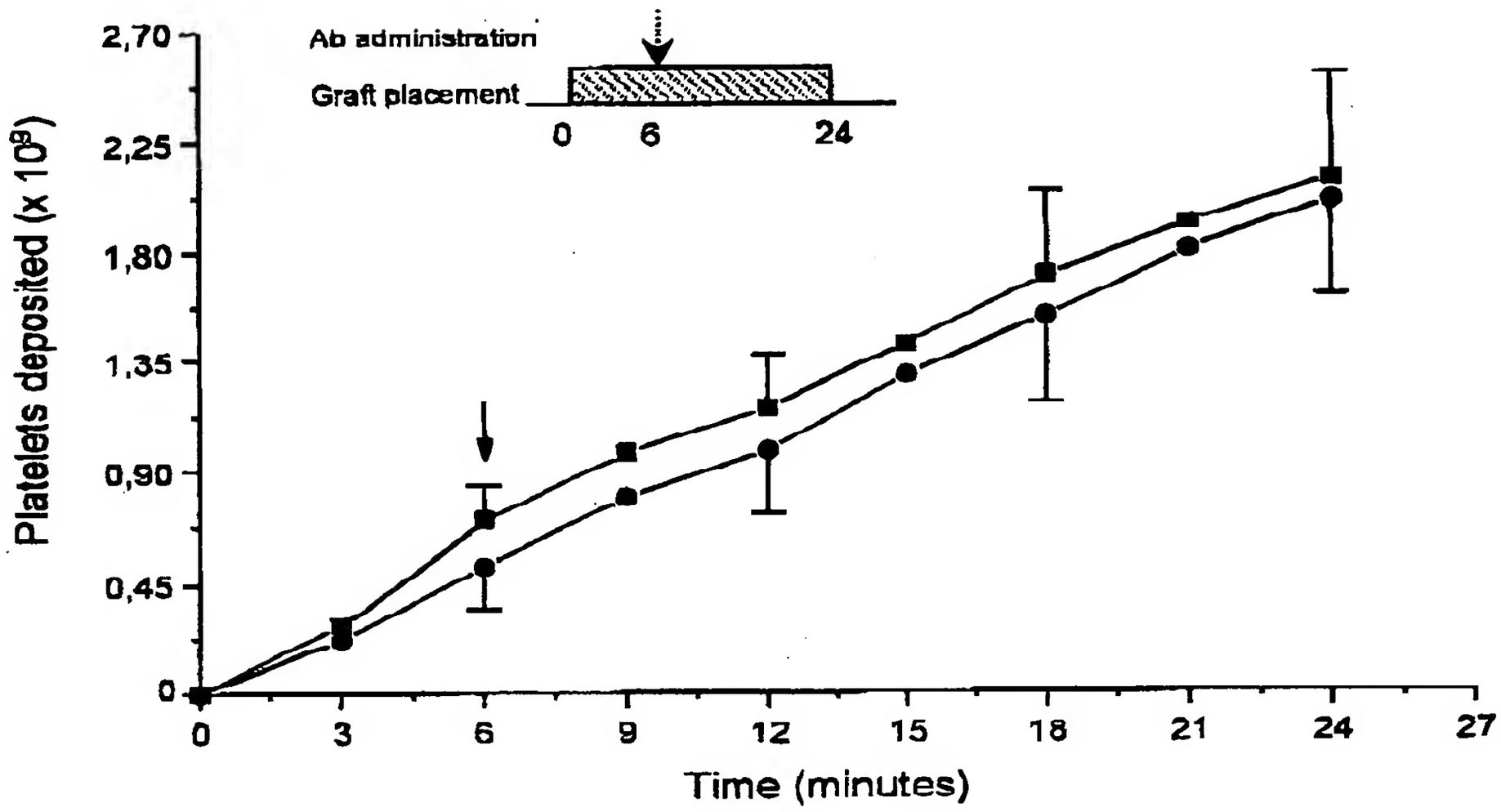

B
Platelets deposited (x 10⁹)
1
2
3
4
0
5
10
15
20
25
30
Time (minutes)

Fig 4


Ab administration
Graft placement
0
6
24
Platelets deposited (x 10⁸)
2,70
2,25
1,80
1,35
0,90
0,45
0
0
3
6
9
12
15
18
21
24
27
Time (minutes)